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Characterization of three inhibitors of endothelial nitric oxide synthase *in vitro* and *in vivo*

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1 Three analogues of L-arginine were characterized as inhibitors of endothelial nitric oxide (NO) synthase by measuring their effect on the endothelial NO synthase from porcine aortae, on the vascular tone of rings of rat aorta and on the blood pressure of the anaesthetized rat.

2 N^G-monomethyl-L-arginine (L-NMMA), N-iminoethyl-L-ornithine (L-NIO) and N^G-nitro-L-arginine methyl ester (L-NAME; all at 0.1–100 μM) caused concentration-dependent inhibition of the Ca^{2+}-dependent endothelial NO synthase from porcine aortae.

3 L-NMMA, L-NIO and L-NAME caused an endothelium-dependent contraction and an inhibition of the endothelium-dependent relaxation induced by acetylcholine (ACh) in aortic rings.

4 L-NMMA, L-NIO and L-NAME (0.03–300 mg kg^{-1}, i.v.) induced a dose-dependent increase in mean systemic arterial blood pressure accompanied by bradycardia.

5 L-NMMA, L-NIO and L-NAME (100 mg kg^{-1}, i.v.) inhibited significantly the hypotensive responses to ACh and bradykinin.

6 The increase in blood pressure and bradycardia produced by these compounds were reversed by L-arginine (30–100 mg kg^{-1}, i.v.) in a dose-dependent manner.

7 All of these effects were enantiomer specific.

8 These results indicate that L-NMMA, L-NIO and L-NAME are inhibitors of NO synthase in the vascular endothelium and confirm the important role of NO synthesis in the maintenance of vascular tone and blood pressure.

Introduction

Vascular endothelial cells synthesize nitric oxide (NO) from the terminal guanidino nitrogen atom(s) of L-arginine (Palmer *et al.*, 1988a). This synthesis of NO accounts for the biological actions of endothelium-derived relaxing factor on vascular strips (Palmer *et al.*, 1987; Ignarro *et al.*, 1987; Furchgott, 1990), in perfused hearts (Amezcua *et al.*, 1988; Kelm & Schrader, 1988) and on platelets (Radomski *et al.*, 1987a,b). The formation of NO by vascular endothelial cells in culture is inhibited by N^G-monomethyl-L-arginine (L-NMMA), but not by its D-enantiomer (Palmer *et al.*, 1988b). L-NMMA also inhibits the NO synthase in endothelial cell homogenates (Palmer & Moncada, 1989).

L-NMMA causes an endothelium-dependent increase in the tone of rings of both rabbit aorta (Palmer *et al.*, 1988b; Rees *et al.*, 1989a) and guinea-pig pulmonary artery (Sakuma *et al.*, 1988) and a rise in coronary perfusion pressure in the isolated perfused rabbit (Amezcua *et al.*, 1989) and guinea-pig (Levi *et al.*, 1990) heart. Furthermore, L-NMMA inhibits endothelium-dependent relaxation of rings of rabbit aorta (Palmer *et al.*, 1988b; Rees *et al.*, 1989a) and guinea-pig pulmonary artery (Sakuma *et al.*, 1988) and the release of NO from the perfused rabbit aorta (Rees *et al.*, 1989a). In addition, it inhibits the fall in coronary perfusion pressure induced by acetylcholine (ACh) in the isolated perfused heart of rabbit (Amezcua *et al.*, 1989) and guinea-pig (Levi *et al.*, 1990). These effects of L-NMMA are consistent with inhibition of vascular endothelial NO synthesis from L-arginine under basal and stimulated conditions.

Intravenous administration of L-NMMA induces an increase in mean arterial blood pressure in anaesthetized rabbit (Rees *et al.*, 1989b) and guinea-pig (Aisaka *et al.*, 1989a) and inhibits the hypotension induced by ACh (Rees *et al.*, 1989b) and bradykinin (Aisaka *et al.*, 1989b). Furthermore, the release of NO from perfused aortae taken from L-NMMA-treated rabbits is inhibited (Rees *et al.*, 1989b). Similar observations have been made in man, where an infusion of L-NMMA into the brachial artery significantly reduces forearm blood flow and into the dorsal hand veins inhibits ACh-induced vasodilatation. These effects can be reversed by L-arginine (Vallance *et al.*, 1989a,b). These data indicate that the formation of NO plays an important role in the regulation of blood flow and the control of blood pressure.

In the present study we have examined the effects of L-NMMA *in vitro* and *in vivo* and have characterized the effects of two other L-arginine analogues (Figure 1), N-iminoethyl-L-ornithine (L-NIO) and N^G-nitro-L-arginine methyl ester (L-NAME) as inhibitors of NO synthesis from L-arginine by the vascular endothelium. These compounds have recently been shown to inhibit NO synthase in adrenal

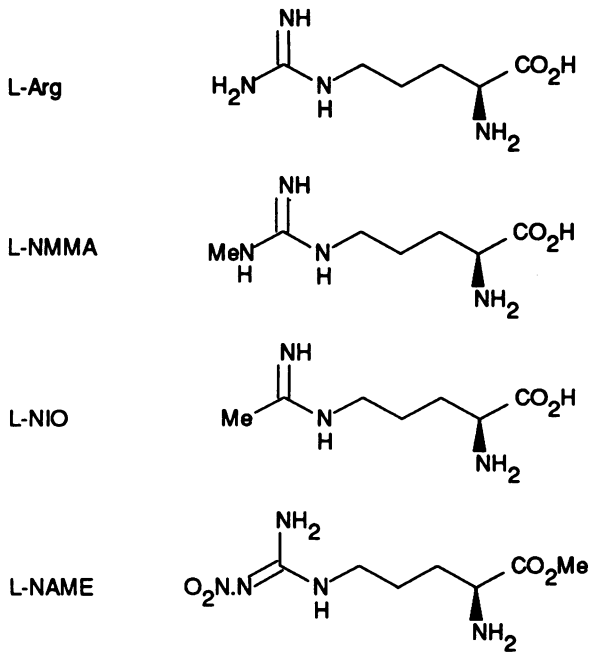

Figure 1 Structural formulae of L-arginine (L-Arg), N^G-monomethyl-L-arginine (L-NMMA), N-iminoethyl-L-ornithine (L-NIO) and N^G-nitro-L-arginine methyl ester (L-NAME).

glands (Palacios *et al.*, 1989) and brain synaptosomes (Knowles *et al.*, 1990). Some of these results were presented at the meeting 'Nitric oxide from L-arginine: a bioregulatory system' at the Royal Society, London, September 14–15, 1989.

Methods

Assay of endothelial nitric oxide synthase

Fresh porcine thoracic aortae, obtained from a local abattoir, were trimmed free of adhering fat and connective tissue and washed with phosphate buffered saline (pH 7.4). The aortae were cut longitudinally and the endothelium removed by scraping with a scalpel. The scrapings were taken up in ice-cold 0.1 M HEPES buffer, pH 7.4, containing 1 mM dithiothreitol (3 aortae/ml) and homogenized by sonication twice for 5 s. The homogenate was centrifuged at 150,000 *g* for 30 min at 4°C and the supernatant incubated with AG50-X8 (Na^+-form; 100 mg ml^{-1} of supernatant) for 5 min at 4°C to deplete endogenous L-arginine. The ion exchange resin was removed by centrifugation at 12,000 *g* for 2 min and the supernatant used as the source of NO synthase and soluble guanylate cyclase.

Nitric oxide synthase was assayed by the stimulation of guanylate cyclase (Knowles *et al.*, 1989) and by the formation of [^{3}H]-citrulline from L-arginine (Palmer & Moncada, 1989). For the determination of guanylate cyclase activity, endothelial homogenate (100 μl) was incubated with NADPH (100 μM), L-arginine (30 μM), Mg^{2+} (5 mM), guanosine 5'-triphosphate (GTP) (1 mM) and the inhibitors in a total volume of 200 μl for 20 min. Incubations were terminated by addition of 50 μl of perchloric acid (20% v/v in H_2O) and neutralised with 100 μl of 1.08 M K_3PO_4. The incubates were then centrifuged (12,000 *g* for 2 min) and aliquots of the supernatant diluted appropriately with 50 mM Tris buffer (pH 7.5) containing 4 mM EDTA. Guanosine 3':5'-cyclic monophosphate (cyclic GMP) was determined by radioimmunoassay.

For determination of [^{3}H]-citrulline formation, incubates contained 5 μCi [^{3}H]-L-arginine in 3 μM L-arginine, NADPH (100 μM) and 100 μl endothelial cytosol in a total volume of 250 μl 0.1 M HEPES buffer, pH 7.4. After incubation for 20 min at 37°C, the reaction was stopped by the addition of 50 μl perchloric acid as above. [^{3}H]-citrulline formation was determined by high performance liquid chromatography (h.p.l.c.) using a μ Bondapak C_{18} column (Waters) with a mobile phase of 2% acetonitrile in 25 mM sodium acetate (pH 4.35) containing sodium hexane sulphonate (15 mM). The radioactivity in the fractions containing [^{3}H]-citrulline was determined by liquid scintillation counting.

Organ bath studies

Male Wistar rats (250–300 g) were killed by a blow on the head and exsanguination. The thoracic aorta was removed, trimmed free of adhering fat and connective tissue and cut into 4 mm rings. The endothelium was removed from some rings by gently rubbing the internal surface with a pipe cleaner. The failure of ACh (1 μM) to induce relaxation of these rings was taken as an indication of endothelium removal.

The rings were mounted under 1.0 g resting tension on stainless steel hooks in 20 ml organ baths filled with Krebs buffer containing indomethacin (5 μM), gassed with 95% O_2 and 5% CO_2 at 37°C. Tension was recorded with Grass FTO3 isometric transducers on a 6-channel multipen recorder (Rikadenki). The tissues were allowed to equilibrate for 60 min during which the Krebs buffer was changed at 15 min intervals, before being contracted submaximally (approx. ED_{90}) by addition of phenylephrine (750 nM). Cumulative relaxation curves to ACh were obtained in each ring to assess the integrity of the endothelium. Rings showing <65% relaxation were discarded. After washout the tissues were allowed to equilibrate for a further 60 min during which the Krebs buffer was changed at 15 min intervals.

In those tissues used to study the effects of the inhibitors on basal tone, L-NMMA, L-NIO or L-NAME (100 μM for each) was added to the organ bath. In a separate series of experiments, cumulative contraction curves to these compounds were obtained in each ring in the presence of a threshold concentration of phenylephrine (10 nM). Those tissues used to study the effects of the inhibitors on ACh-induced relaxation were contracted submaximally by addition of phenylephrine (750 nM). When a stable contraction was obtained L-NMMA, L-NIO or L-NAME was added 10–15 min before a second cumulative relaxation curve to ACh was obtained.

In vivo *studies*

Male Wistar rats (200–300 g) were anaesthetized with sodium thiobutabarbitone (120 mg kg^{-1}, i.p.). A tracheotomy was performed and a catheter containing heparinised (10 units ml^{-1}) saline inserted into the right carotid artery for the measurement of mean arterial blood pressure. A catheter was also inserted into the femoral vein for continuous infusion of phenylephrine (150–300 μg kg^{-1}) and a 25 gauge butterfly needle inserted into the tail vein for administration of all other drugs. Anaesthesia was then maintained by bolus administration of sodium thiobutabarbitone (5–10 mg kg^{-1}, i.v.). Indomethacin (5 mg kg^{-1}, i.v.) was administered 5 min prior to the experimental protocol and all drugs were administered in 0.9% NaCl. The hypotension induced by the vasodilators was measured as the area comprising the fall and duration of the response and determined by computerized planimetry.

Chemicals

Acetylcholine bromide, NADPH, L- and D-arginine (free base), indomethacin, bradykinin acetate, phenylephrine hydrochloride, L-NAME hydrochloride (all Sigma), dithiothreitol (Boehringer, Mannheim), AG50-X8 (Bio Rad), sodium thiobutabarbitone (Inactin, Byk-Gulden, Konstanz, FRG), sodium hexane sulphonate (BDH) and L[^{3}H]-arginine (Amersham) were obtained as indicated. L-NMMA acetate, L-NIO hydrochloride, their D-enantiomers and D-NAME hydrochloride were synthesized as described previously (Patthy *et al.*, 1977; Scannell *et al.*, 1972).

Statistics

Results are expressed as mean ± s.e.mean for n separate experiments. Student's paired or unpaired t test, as appropriate, was used to determine the significance of differences between means and $P < 0.05$ was taken as statistically significant. The EC_{50}/IC_{50} responses were expressed as a percentage of the maximum effect of each agonist from computer-constructed sigmoid logistic dose-response curves.

Results

Studies in vitro

Inhibition of nitric oxide synthase Cyclic GMP formation in endothelial cytosol was stimulated from 1.54 ± 0.16 to 35.55 ± 2.88 pmol min^{-1} mg^{-1} protein ($n = 10$) by L-arginine (30 μM: EC_{50} 18.8 ± 2.6 μM; $n = 3$). This was dependent on the presence of NADPH (100 μM; $n = 6$). Endothelial cytosol, in the presence of NADPH (100 μM), also formed [^{3}H]-citrulline (43.9 ± 7.6 fmol min^{-1} mg^{-1} protein; $n = 10$) from [^{3}H]-L-arginine (3 μM).

The formation of cyclic GMP in the presence of L-arginine (30 μM) was inhibited by EGTA (0.001–1 mM; IC_{50} = 46.0 ± 7.1 μM; $n = 3$) and by EDTA (0.001–1 mM; IC_{50} = 46.8 ± 10.4 μM; $n = 5$). The formation of [^{3}H]-citrulline in the presence of L-arginine (3 μM) was also inhibited by these concen-

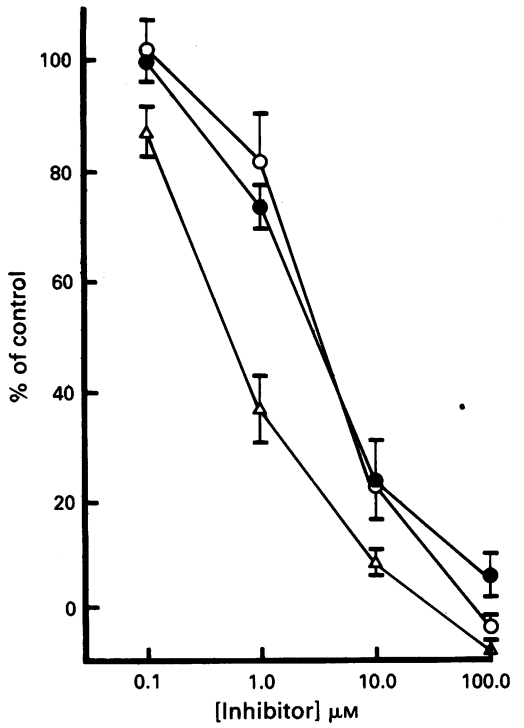

Figure 2 Effects of L-NMMA (●), L-NIO (△), and L-NAME (○) on the stimulation of soluble guanylate cyclase in endothelial cytosol in the presence of L-arginine (30 μM) and NADPH (100 μM). Data are expressed as % inhibition of control (35.55 ± 2.88 pmol min^{-1} mg^{-1} protein, $n = 10$). Each point is the mean of 4 experiments with s.e.mean shown by vertical bars.

trations of EGTA (IC_{50} = 40.8 ± 7.2 μM; $n = 5$) and EDTA (IC_{50} = 45.0 ± 12.0 μM; $n = 5$).

L-NMMA, L-NIO and L-NAME (all at 0.1–100 μM; Figure 2) in the presence of L-arginine (30 μM) and NADPH (100 μM), inhibited cyclic GMP formation by endothelial cytosol with IC_{50}s of 2.9 ± 0.2, 0.5 ± 0.1 and 3.1 ± 0.4 μM ($n = 4$ for each) respectively. The stimulation of soluble guanylate cyclase by sodium nitroprusside (100 μM; 705.6 ± 60.9 pmol min^{-1} mg^{-1} protein; $n = 4$) was not affected by L-NMMA, L-NIO or L-NAME (100 μM; $n = 3$ for each). These compounds also inhibited the formation of [^{3}H]-citrulline in the presence of L-arginine (3 μM) and NADPH (100 μM) with IC_{50}s of 0.092 ± 0.08, 0.015 ± 0.01 and 0.090 ± 0.004 μM ($n = 4$ for each) respectively.

Effects on vascular rings L-NMMA, L-NIO and L-NAME (100 μM) alone induced a small but significant endothelium-dependent contraction of rings of rat aorta of 5.5 ± 0.5, 11.8 ± 1.5 and 5.2 ± 0.6% respectively (n = 17–19) of that induced by phenylephrine (750 nM; tension approx. 3 g). In the presence of a threshold concentration of phenylephrine (10 nM), however, these compounds (0.1–300 μM) induced a greater endothelium-dependent contraction of the rings (Figure 3). The EC_{50}s for the three compounds were 12.5 ± 1.3, 2.1 ± 0.6 and 26 ± 6 μM, respectively (n = 6–8). The maximum contraction induced by L-NMMA, L-NIO and L-NAME was 40.5 ± 6.5, 92.3 ± 9.7 and 39.7 ± 7.4% respectively (n = 6–8) of that induced by phenylephrine (750 nM). The contractions induced by equieffective concentrations of L-NMMA (10 μM) and L-NIO (0.3 μM) were rapid in onset and reached a plateau in 8.3 ± 2.4 and 8.2 ± 2.3 min ($n = 3$ for each), whereas L-NAME (10 μM) produced a slower response which only reached a plateau in 15.7 ± 1.9 min ($n = 3$). Their D-enantiomers had no effect (100 μM; $n = 3$ for each).

Acetylcholine (0.01–3 μM) caused endothelium-dependent relaxation of rings of rat aorta contracted (approx. EC_{90}) with phenylephrine (750 nM). The maximum relaxation observed was 86.7 ± 1.7% ($n = 8$) of the contraction induced by phenylephrine. The relaxation induced by ACh was inhibited in a concentration-dependent manner by L-NMMA (1–1000 μM), L-NIO (0.3–30 μM) and L-NAME (0.1–10 μM) with IC_{50}s of 9.5 ± 1.1, 2.0 ± 0.3 and 0.54 ± 0.04 μM ($n = 3$ for each) respectively (Figure 4). The maximum degree of inhibition of ACh-

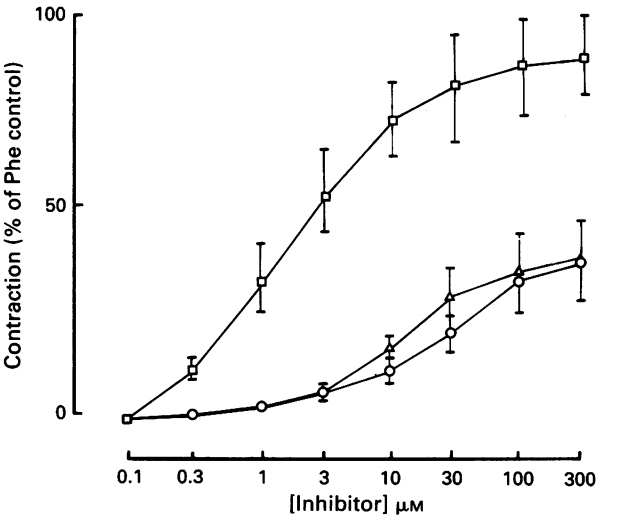

Figure 3 Contraction of rings of rat aorta induced by L-NMMA (△), L-NIO (□) and L-NAME (○) in the presence of 10 nM phenylephrine, expressed as a % of the contraction induced by phenylephrine (Phe, 750 nM). Each point is the mean of 6–8 experiments with s.e.mean shown by vertical bars.

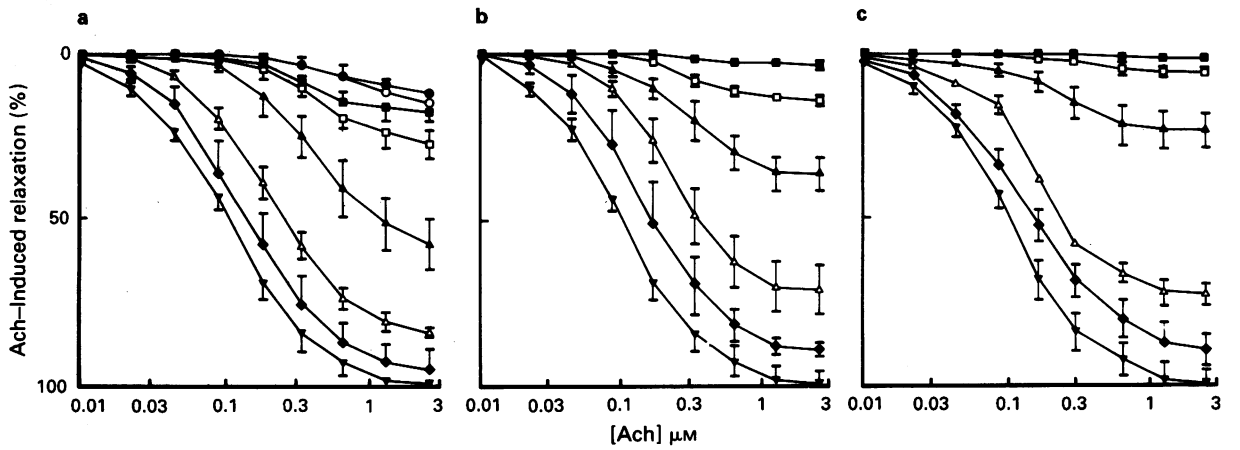

Figure 4 Inhibition by (a) L-NMMA (1–1000 μM), (b) L-NIO (0.3–30 μM) and (c) L-NAME (0.1–10 μM) of the relaxation of rat aortic rings induced by acetylcholine (ACh), expressed as a % of the maximum relaxation induced by ACh. In (a), (▼) control; (◆) 1; (△) 3; (▲) 10; (□) 30; (■) 100; (○) 300 and (●) 1000 μM L-NMMA. In (b), (▼) control; (◆) 0.3; (△) 1; (▲) 3; (□) 10 and (■) 30 μM L-NIO. In (c), (▼) control; (◆) 0.1; (△) 0.3; (▲) 1; (□) 3 and (■) 10 μM L-NAME. Each point is the mean of 3 experiments with s.e.mean shown by vertical bars.

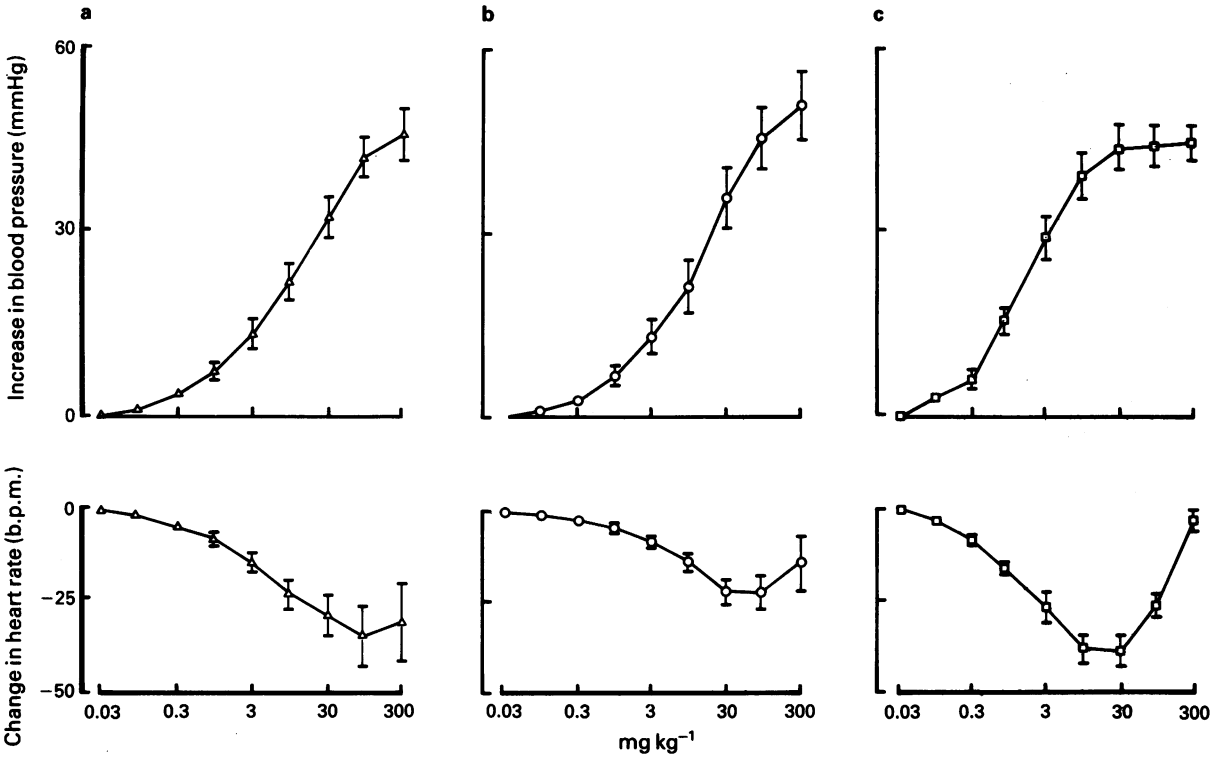

Figure 5 Effect of (a) L-NMMA, (b) L-NIO and (c) L-NAME (0.03–300 mg kg^{-1}, i.v. for each) on mean arterial blood pressure and heart rate in anaesthetized rats. The resting blood pressures were 104.6 ± 8.2 (n = 7), 102.9 ± 2.6 (n = 8) and 111.3 ± 2.6 mmHg (n = 6) for L-NMMA-, L-NIO- and L-NAME-treated rats respectively. The resting heart rates were 366 ± 16 (n = 7), 362 ± 11 (n = 8) and 372 ± 6 (n = 6) b.p.m. respectively. Each point is the mean of 6–8 experiments with s.e.mean shown by vertical bars.

induced relaxation by L-NMMA (1000 μM), L-NIO (30 μM) and L-NAME (10 μM) was 89.4 ± 1.1, 96.6 ± 1.3 and 98.3 ± 0.2% (n = 3 for each) respectively. Their D-enantiomers were without effect (100 μM; n = 3).

In vivo

L-NMMA, L-NIO and L-NAME (0.03–300 mg kg^{-1}, i.v.), but not their D-enantiomers (100 mg kg^{-1}, i.v.), caused a dose-dependent increase in mean arterial blood pressure (Figure 5) with EC_{50}s of 16.7 ± 4.4, 19.5 ± 5.8 and 2.4 ± 0.4 mg kg^{-1} (n = 6–8) respectively. The maximum increases in blood pressure produced by L-NMMA, L-NIO and L-NAME (300 mg kg^{-1}, i.v. for each) were 45.6 ± 4.4 (n = 7), 50.7 ± 5.5 (n = 8) and 44.1 ± 3.1 mmHg (n = 6) and were not significantly different from each other. The increase in blood pressure was rapid in onset for L-NMMA and L-NIO (10 mg kg^{-1}, i.v. for both) and reached a plateau in 4.2 ± 1.1 and 3.5 ± 0.8 min (n = 4) respectively, whereas L-NAME (1 mg kg^{-1}, i.v.) produced a slower increase in blood pressure and reached a plateau in 6.0 ± 0.4 min (n = 6). The dose-dependent increase in blood pressure induced by L-NMMA,

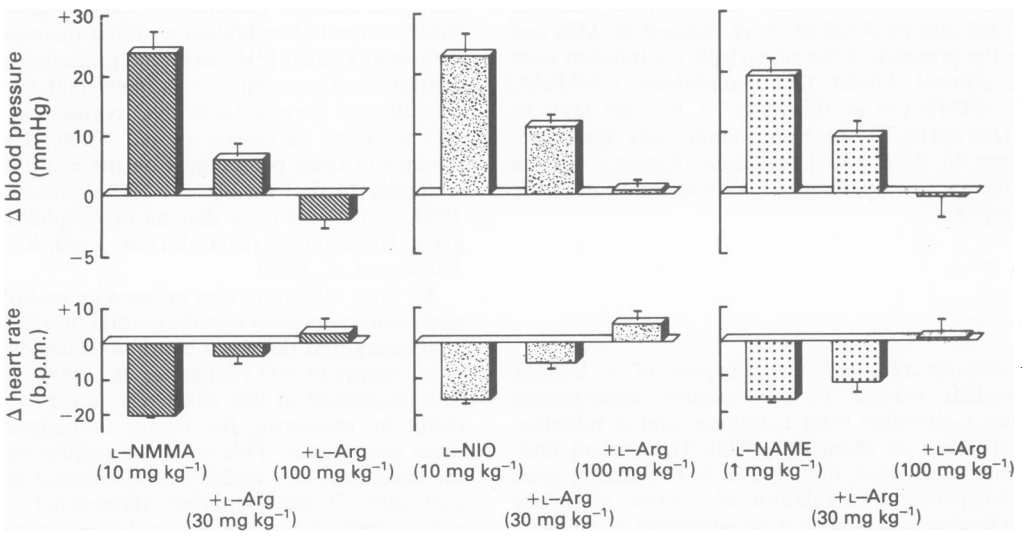

Figure 6 Reversal by L-arginine (L-Arg, 30 and 100 mg kg^{-1}, i.v.) of the effects of L-NMMA (10 mg kg^{-1}, i.v.), L-NIO (10 mg kg^{-1}, i.v.) and L-NAME (1 mg kg^{-1}, i.v.) on blood pressure and heart rate in the anaesthetized rat. The resting blood pressures were 98.6 ± 5.3 (n = 4), 105 ± 5.3 (n = 4) and 108.4 ± 4.4 (n = 6) mmHg respectively and the corresponding resting heart rates were 381 ± 15 (n = 4), 385 ± 13 (n = 4) and 377 ± 14 (n = 6) b.p.m. Each column is the mean of 4–6 experiments with s.e.mean shown by vertical bars.

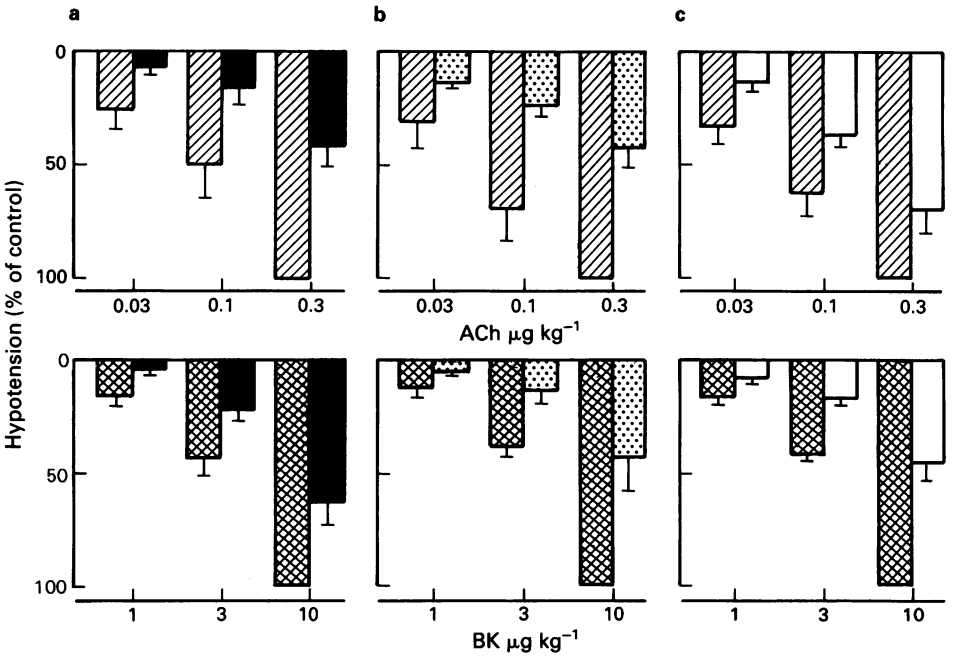

Figure 7 Inhibition by (a) L-NMMA (solid columns), (b) L-NIO (stippled columns) and (c) L-NAME (open columns) (100 mg kg^{-1} for each) of the hypotensive response to acetylcholine (ACh, hatched columns 0.03–0.3 μg kg^{-1}, i.v.) and bradykinin (BK, cross-hatched columns 1–10 μg kg^{-1}, i.v.) in anaesthetized rats. The hypotension was measured as the area comprising the fall and the duration of the response and was expressed as a % of the maximum control response. Each column is the mean of 4–5 experiments with s.e.mean shown by vertical bars.

L-NIO and L-NAME was accompanied by a dose-dependent bradycardia which tended to reverse at higher doses (Figure 5).

Administration of L-arginine (30–100 mg kg^{-1}, i.v.), which had no direct effect either on mean arterial blood pressure or on heart rate, reversed in a dose-dependent manner the increases in blood pressure and bradycardia produced by L-NMMA (10 mg kg^{-1}, i.v., $n = 4$), L-NIO (10 mg kg^{-1}, i.v., $n = 4$) and L-NAME (1 mg kg^{-1}, i.v., $n = 6$) (Figure 6).

Acetylcholine (0.03–0.3 μg kg^{-1}) and bradykinin (1–10 μg kg^{-1}) caused a dose-dependent fall in mean arterial blood pressure. The absolute falls in mean arterial blood pressure induced by ACh (0.3 μg kg^{-1}) and bradykinin (10 μg kg^{-1}) were 47.9 ± 3.4 ($n = 15$) and 51.2 ± 3.1 mmHg ($n = 12$) respectively. Phenylephrine (150–300 μg kg^{-1} h^{-1}) was used to raise the blood pressure to a level similar to that induced by L-NMMA, L-NAME and L-NIO (all at 100 mg kg^{-1}); the falls in blood pressure induced by ACh and bradykinin in the presence of the phenylephrine infusion were taken as the control. Under these conditions, L-NMMA, L-NIO and L-NAME (all at 100 mg kg^{-1}), but not their D-enantiomers (100 mg kg^{-1}), inhibited significantly the hypotensive response to ACh and bradykinin (Figure 7), when measured as the area comprising the fall and duration of the hypotensive response.

Discussion

We have previously reported the presence of a soluble, NADPH-dependent enzyme in the vascular endothelium which generates L-citrulline from L-arginine and is inhibited by L-NMMA (Palmer & Moncada, 1989). The present findings support our conclusion that this is NO synthase, since the L-arginine-dependent stimulation of soluble guanylate cyclase, which is now accepted as a measurement of NO formation (Knowles *et al.*, 1989; Mülsch *et al.*, 1989), is also dependent on NADPH and is inhibited by L-NMMA.

Furthermore, the elevation of cyclic GMP and the formation of [^{3}H]-citrulline are inhibited by EGTA and EDTA over the same range of concentrations, indicating that endothelial NO synthase is Ca^{2+}-dependent, since this is the only divalent cation for which both chelating agents have similar affinities (Dawson *et al.*, 1986). These data are in agreement with a recent report (Meyer *et al.*, 1989) and show that this NO synthase is similar to that in the brain (Knowles *et al.*, 1989) and in the adrenal gland (Palacios *et al.*, 1989) in terms of their dependence on this divalent cation.

The endothelial NO synthase is also inhibited in an enantiometrically specific manner by L-NIO and L-NAME which have recently been characterized as inhibitors of NO synthase in the adrenal gland (Palacios *et al.*, 1989) and brain (Knowles *et al.*, 1990). L-NIO was approximately 5 times more potent as an inhibitor of endothelial NO synthase than the other arginine analogues. Since none of these compounds affected the rise in cyclic GMP induced by sodium nitroprusside, the data indicate that these compounds inhibit NO synthase rather than the soluble guanylate cyclase. Furthermore, since all these compounds exhibited a similar increase in potency when measured against [^{3}H]-citrulline formation from a lower concentration of L-arginine, it is likely that they are competitive inhibitors of the endothelial NO synthase as they are for brain NO synthase (Knowles *et al.*, 1990). The rank order of potency of these compounds on the endothelial NO synthase is similar to that on platelet NO synthase (Radomski *et al.*, 1990), but differs from that on neutrophil, brain and adrenal gland NO synthase (McCall *et al.*, 1990; Knowles *et al.*, 1990; Palacios *et al.*, 1989).

All three inhibitors also induced an endothelium-dependent and enantiomerically specific contraction of the vascular rings, confirming that there is a continuous use of L-arginine for the basal release of NO (Palmer *et al.*, 1988b; Rees *et al.*, 1989a). The magnitude of this basal release can be determined indirectly by measuring the degree of contraction induced by these compounds. This could be readily demonstrated when the inhibitors were added in the presence of a threshold concentration of phenylephrine. Interestingly, L-NIO, which is not a substituted guanidino analogue of L-arginine, was again approximately 5–10 times more potent than L-NMMA and L-NAME and the contraction induced by L-NIO reached a significantly greater maximum. Since this effect was enantiomerically specific it is likely that it is due solely to an effect on NO synthesis.

The endothelium-dependent relaxations induced by ACh were inhibited by all three compounds by at least 90%. Previous studies have shown that in rings of rabbit aorta (Rees *et al.*, 1989a) and guinea-pig pulmonary artery (Sakuma *et al.*, 1988) L-NMMA only inhibits ACh-induced relaxation by approximately 65%. However, N^G-nitro-L-arginine inhibits the ACh-induced relaxation in the rabbit aorta by more than 90% (Kobayashi & Hattori, 1990) and in the rabbit femoral artery by about 80% (Mülsch & Busse, 1990). These data suggest that the difference in the maximum effect in different preparations is probably due to variations in the uptake and metabolism of these compounds by endothelial cells, rather than to the involvement of another mediator in endothelium-dependent relaxation.

All three compounds inhibited endothelium-dependent relaxation induced by ACh and caused an increase in blood pressure, with L-NAME being more potent than L-NIO and L-NMMA in both systems. These findings are consistent with previous reports that N^G-nitro-L-arginine is more potent than L-NMMA in other vascular preparations *in vitro* (Mülsch & Busse, 1990; Moore *et al.*, 1990). This rank order of potency differs from that for inhibition of NO synthase and for their contractile activity on rings of rat aorta. This difference is largely attributable to the substantial increase in potency of L-NAME relative to the other two analogues, the reasons for which are not clear. An interesting possibility suggested by Mülsch *et al.* (1989) is that there are two NO synthases in the vascular endothelium, one Ca^{2+}-dependent and one Ca^{2+}-independent. If one is agonist-activated and is more sensitive to L-NAME, then this would explain the increased potency of this compound against ACh-induced relaxation. Further work is required to clarify these differences.

The rate of onset of the hypertensive response to all three compounds was similar to that of the increase in basal tone of rings *in vitro*, further indicating that the hypertensive response is mediated by inhibition of basal endothelial NO synthesis. The maximum increase in blood pressure induced by all three compounds was similar. Therefore, the level of increase in blood pressure achieved probably represents the total vasodilator tone produced by NO.

The bradycardia which accompanies the elevation in blood pressure was reversed at higher doses of the compounds, particularly with L-NAME. An L-arginine:NO pathway has been implicated in neurotransmission in non-adrenergic, non-cholinergic preparations (Gillespie *et al.*, 1989; Gibson *et al.*, 1990) and in the brain (Knowles *et al.*, 1989; Garthwaite *et al.*, 1989). The accompanying bradycardia may therefore involve a neuronal L-arginine:NO pathway.

L-Arginine has no direct effect on blood pressure or heart rate, indicating that its availability is not rate-limiting for basal NO synthesis in the rat and confirming previous reports for other species (Rees *et al.*, 1989b; Aisaka *et al.*, 1989a). The increase in blood pressure and the accompanying bradycardia induced by equi-effective doses of L-NMMA, L-NIO and L-NAME were fully reversed following administration of L-arginine. Since L-NAME is about 10 fold more potent than L-NMMA and L-NIO in increasing blood pressure, a 10 fold greater molar excess of L-arginine is required to reverse its effects fully. This is consistent with these compounds inhibiting NO synthesis by competing with the substrate.

In contrast to the effect of L-NMMA on hypotension induced by ACh in the rabbit (Rees *et al.*, 1989b), the inhibitory effect of L-NMMA, L-NIO and L-NAME on ACh- and bradykinin-induced hypotension in the rat was only observed when measured in terms of fall and duration, rather than fall alone, confirming similar findings in the guinea-pig (Aisaka *et al.*, 1989b). These authors proposed that the ACh-induced hypotension in the guinea-pig is the resultant of two phases, an immediate vasodilatation, which is resistant to blockade by L-NMMA, and a longer-lasting vasodilatation, which is susceptible to blockade by L-NMMA. They therefore suggest that only the latter requires the synthesis of NO from L-arginine. However, *in vitro* studies (Giles *et al.*, 1990) have recently shown that resistance to blockade by L-NAME is more apparent in vascular preparations with a highly efficient receptor-effector coupling, such as when ACh is a potent full agonist. Indeed, the finding that L-NMMA inhibits the initial fall in blood pressure induced by ACh in the rabbit (Rees *et al.*, 1989b), where the potency of ACh is 10 fold lower than in the rat, is consistent with this explanation.

In summary, L-NMMA, L-NIO and L-NAME are inhibitors of the formation of NO from L-arginine by the vascular endothelium, which behave in a qualitatively similar pharmacological manner *in vitro* and *in vivo*. Although all their effects are consistent with inhibition of NO synthase, there are differences in potency of these compounds in different systems which require further investigation. Furthermore, the activity of these compounds *in vitro* and *in vivo* confirms the important role of vascular endothelial NO synthesis in regulating blood pressure and flow. Pharmacokinetic and metabolic studies on these compounds and characterization of the distribution and activity of the L-arginine:NO pathway at the different levels of the vascular tree are now required.

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